REMARKS

The Office Action mailed November 18, 2002, has been received and carefully reviewed. Claims 63-76 are withdrawn from consideration, and claims 77-90 are examined. Reconsideration and withdrawal of the rejections of the claims of the above-identified application is respectfully requested.

Two of the references filed with the IDS on October 31, 2002 were not in English. Enclosed herewith are an English translation of the entire document WO 98/26294, and an English translation of the claims of WO 97/00447 (from the EP equivalent EP0834076).

Rejections Under 35 U.S.C. §112, First Paragraph

Claims 77-90 are rejected as not enabled by the specification. The Examiner acknowledges that the prior art does not teach the claimed invention, and asserts that the specification fails to teach two specific epitope regions on the TSHR or TSHR fragment or how to generate antibodies that bind only one of the specific regions. Applicants respectfully traverse the rejection.

✱ The specification teaches, in examples 1-5 on pages 8-10, how to produce an antibody that binds TSH receptor at the same time as TSH, indicating TSH receptor has at least two distinct epitope regions, with the antibody binding one region and TSH binding the other region.

Example 1 describes the preparation of cDNA clones for the full-length porcine TSH receptor. RNA was cloned from porcine thyroid tissue and the mRNA was purified. This mRNA was then employed to synthesize a cDNA library from which cDNA clones for full-length porcine TSH receptor were obtained and fully sequenced. The described preparatory techniques of Example 1 were well known in the art at the time the invention was made and would be readily reproducible by a skilled artisan without undue burden.

Example 2 describes the preparation of a stable cell line for expressing the TSH receptor and thus the provision of recombinant TSH receptor. Again the techniques described

were well known in the art at the time the invention was made and would be readily reproducible by a skilled artisan without undue burden.

Example 3 describes the preparation of detergent solubilized recombinant porcine TSH receptor expressed by a stable cell line prepared per the teaching of Example 2 as discussed above. Again the techniques were well known in the art at the time the invention was made and sufficiently described so as to be reproducible by a skilled artisan without undue burden.

Example 4 describes the preparation of a fusion protein, wherein the 3' end of cDNA (1809 to 2295bp encoding the last 160 amino acids of the TSH receptor) prepared as above was cloned in frame with glutathione S-transferase (GST) fusion protein in pGEX2T vector. An *E. coli* culture was transformed with the pGEX2T/TSH receptor plasmids and expression of the TSH receptor / GST fusion proteins were carried out. Polyacrylamide electrophoresis was carried out and the expressed TSH receptor / GST fusion proteins were then electroeluted from polyacrylamide gel slices, dialyzed and stored. Again the techniques were well known in the art at the time the invention was made and sufficiently described so as to be reproducible by a skilled artisan without undue burden.

Example 5 describes immunization of BALB C mice with electroeluted TSH receptor / GST fusion protein until the titer of antibody was high. Mouse spleen cells were then fused with mouse myeloma cells and cloned to produce stable hybridomas secreting TSH receptor antibody using standard techniques. Antibody Mab 4E31 was then purified from hybridoma culture supernatants. Again the techniques were well known in the art at the time the invention was made and sufficiently described so as to be reproducible by a skilled artisan without undue burden.

A skilled artisan reading the above referred to Examples in the specification would readily have understood the well-known genetic engineering and immunology

techniques employed. The techniques described represent a recognized sequence of genetic engineering and immunology techniques for the preparation of a recombinant antigenic molecule and antibody, namely: construction of a cDNA library and isolation of cDNA clones coding the antigenic molecule; preparation of a stable cell line for expressing the cDNA clones and thus the provision of recombinant antigenic molecule; immunization of mice with the recombinant antigenic molecule and the subsequent isolation of an antibody that specifically binds the antigenic molecule.

Further to the teaching of the present invention as provided by the specification, the above techniques would have been routine for an artisan to follow to reproduce the preparation of a recombinant protein corresponding to the C terminal portion of the TSH receptor, namely the last 160 amino acids of the TSH receptor encoded by cDNA base pairs 1809 to 2295, to subsequently immunize a population of mice with recombinant protein and to isolate an antibody raised in response thereto. To illustrate this, Applicants have reproduced the above referred to Examples 1 to 5 and have prepared a second antibody, Mab 8B7, in response to the recombinant fusion protein as taught by the Examples. Applicants have further characterized the binding properties for Mab 8B7 and have found that Mab 8B7 binds to an epitope region of the TSH receptor distinct from an epitope region recognized by autoantibodies to the TSH receptor. Thus, Applicants have shown the preparation and characterization of an additional antibody that binds a second epitope region of the TSH receptor as required by the claims, by following the techniques provided in the specification.

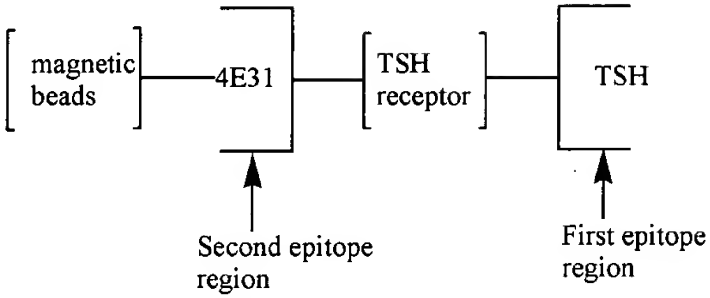
Applicants enclose a declaration by Dr. Bernard Rees Smith to substantiate the above, detailing the preparation and characterization of Mab 8B7. As evidenced by the successful production of an additional antibody by following the examples, the specification provides sufficient enablement whereby a skilled artisan would be able to prepare a recombinant protein and antibody. The skilled artisan would recognize that the recombinant protein would include an epitope region of the TSH receptor distinct from the autoantibody-binding epitope. No further clarification regarding this second epitope region would be necessary for the artisan to be in a position to practice the present invention as defined in the claims of the specification. Applicants submit that the skilled

artisan would be able to practice the invention as defined in the claims, without undue burden or further experimentation, other than following the examples provided by the specification.

Applicants would like to make clear, however, that while the above techniques as described in the Examples are well known techniques and are provided in sufficient detail by the Examples to enable a skilled artisan to reproduce the Examples, this does not in any way detract from the inventiveness of the subject matter defined in the claims. To the contrary, the key to the present invention is the preparation of an antibody which binds to an epitope region distinct from the epitope region involved in TSH or TSH receptor autoantibody binding. The provision of this antibody in turn leads to methods and kits according to the present invention, whereby a TSH receptor or fragment thereof can be stably bound to a suitable support via the thus prepared antibody or fragment thereof. The TSH or TSH receptor autoantibody binding can then be monitored at a distinct epitope region of the TSH receptor, using methods and kits according to the present invention. This was in no way envisaged or suggested by the prior art, as indeed has been acknowledged by the Examiner.

With respect to the first and second epitope regions of a TSH receptor as defined in the claims of the specification, Applicants have characterized the antibody binding respectively associated with these epitope regions and this is further illustrated by Example 6 of the specification. More particularly, having isolated antibody Mab 4E31, Applicants then immobilized Mab 4E31 to beads or tubes and solubilized TSH receptor was then bound to Mab 4E31 and the binding of the thus immobilized [Mab 4E31 - TSH receptor] with either TSH or TSH receptor autoantibodies investigated.

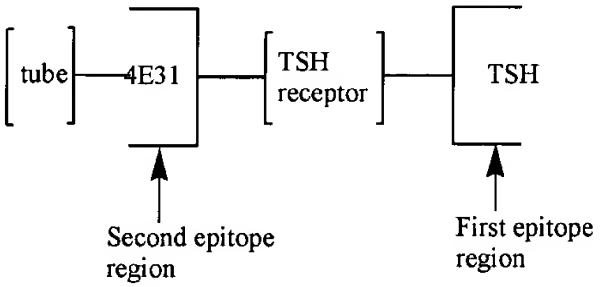
Table 1 of Example 6 clearly shows the binding of the immobilized recombinant TSH receptor to both TSH and Mab 4E31. In other words, the following complex was formed immobilized to magnetic beads:



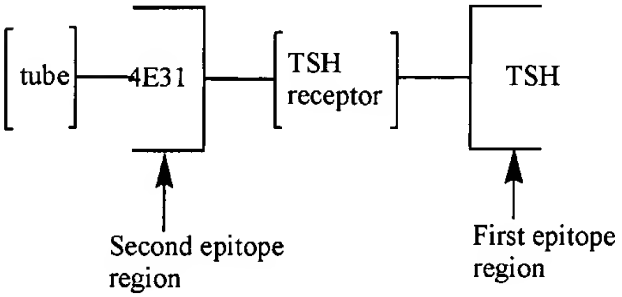
The presence of first and second epitope regions is, therefore, clearly illustrated by the results of Table 1.

Table 2 of Example 6 shows inhibition of TSH binding to recombinant TSH receptor by autoantibodies present in sera from patients with Graves' disease. It is well documented in the art that TSH receptor autoantibodies mimic the normal action of TSH, thus binding to the TSH binding site of the TSH receptor. In other words, TSH receptor autoantibodies inhibit binding of TSH to the first epitope region of the TSH receptor as illustrated above and as such it will be understood from the results given in Table 2 that the following complexes form:

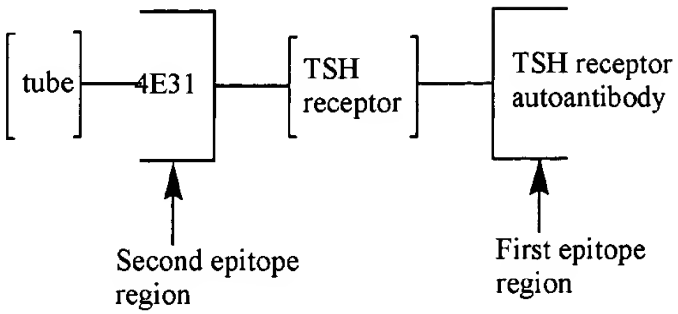
(i) For healthy donor sera, sera from patients with Hashimoto's thyroiditis and patients with systemic lupus erythematosus:



(ii) For sera with patient's with Graves disease, both
(a)



and
(b)



where complexes of (b) compete with the formation of complexes of (a) due to the presence of TSH receptor autoantibodies.

The presence of first and second epitope regions is, therefore, again clearly illustrated by the results of Table 2. Table 3 of Example 6 shows similar results to those illustrated by Table 2 as discussed above, but employing native porcine receptor. Table 4 of Example 6 shows that inhibition of TSH binding is dose dependent on the concentration of TSH receptor autoantibodies, by use of a standard thyroid stimulating antibody preparation, namely International Standard 90/672.

In summary, Applicants submit that:

(i) the specification teaches the isolation of an antibody to the TSH receptor, Mab 4E31, which interacts with an epitope region of the TSH receptor distinct from that for autoantibody binding;

(ii) more precise definition of the epitope regions involved in the above binding is not required for enablement or practice of the present invention;

(iii) the disclosure of the specification is enabling for the scope of the claims, as is further illustrated by the Applicants' preparation of Mab 8B7 and the use thereof in detecting the presence of autoantibodies (detailed in the declaration provided by Dr. Bernard Rees Smith);

(iv) the specification clearly illustrates the presence of first and second epitope regions on the TSH receptor (by virtue of the described TSH receptor autoantibody or TSH binding to the first epitope region, and antibody Mab 4E31 binding to the second epitope region), and the use thereof in methods and kits of the present invention.

For the reasons set forth above, Applicants submit that the instant specification does enable one of ordinary skill in the art to practice the claimed invention. Withdrawal of the rejection is respectfully requested.

It is respectfully submitted that each of the presently pending claims are in condition for allowance and notification to that effect is respectfully requested. The Examiner is invited to contact Applicants' representative at the below-listed telephone number, if it is believed that prosecution of this application may be assisted thereby.

Respectfully submitted,

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DE19522171 [redacted]

Competitive immunoassay for auto-antibodies against thyroid stimulating hormone receptor

Patent Number: DE19522171

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EC Classification: C07K14/72B, G01N33/564, G01N33/76

Equivalents: JP11507727T, WO9700447

Abstract

Determn. of anti-TSH (thyroid stimulating hormone) receptor auto-antibodies (Ab) in human serum or plasma comprises (1) incubating sample with solubilised TSH receptor and labelled TSH; (2) transferring the receptor, with bound fraction of Ab and labelled TSH, to a solid phase and removal of the liq. phase, and (3) measuring the amount of label in the solid phase. The new feature is that in prepn. of the reaction mixt., TSH and the receptor are added almost simultaneously and the mixt. is subjected to a single-stage incubation. In a modification, the fraction of TSH not bound to receptor is converted to solid phase and measured.

feste Phase und deren Abtrennung von der flüssigen Phase, und
- Messung der Menge des in der festen Phase gebundenen markierten TSH anhand der für das verwendete Label typischen physikalischen oder chemischen Nachweismethode,

dadurch gekennzeichnet, daß man

- als TSH-Rezeptor ein durch Extraktion und Solubilisierung aus einem humanen oder tierischen Schilddrüsenmaterial gewonnenes TSH-Rezeptor-Präparat oder einen solubilisierten rekombinanten TSH-Rezeptor verwendet,
- als markiertes TSH ein radioaktiv markiertes TSH verwendet, das durch direkte Radiolodierung eines durch Aufreinigung aus crudem bovinem TSH gewonnenen TSH-Präparats, das eine biologischen Aktivität von mehr als 40 IE TSH/mg Protein aufweist und erhältlich ist durch affinitätschromatographische Reinigung von handelsüblichem crudem bovinem TSH an einer anti-TSH-Antikörper-Säule und anschließende Ionenaustausch-Chromatographie an einem schwach sauren Kationenaustauscher mit endständigen Carboxylgruppen auf der Basis eines polyamidbeschichteten Kieselgels, erhalten wurde, und
- zur Bereitung der Reaktionsmischung die Probe, das markierte TSH-Präparat und den TSH-Rezeptor im wesentlichen gleichzeitig zusammengibt und die so erhaltene Reaktionsmischung einer einstufigen Inkubation unterwirft.

2. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß man zur Bereitung der Reaktionsmischung zuerst die Probe und das markierte TSH-Präparat zusammengibt und die Reaktion durch anschließende Zugabe des TSH-Rezeptors startet.

3. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß man ein porcines TSH-Rezeptor-Präparat oder einen rekombinanten humanen TSH-Rezeptor verwendet.

4. Verfahren nach einem der vorausgehenden Ansprüche, dadurch gekennzeichnet, daß man den TSH-Rezeptor mit den daran gebundenen Anteilen an anti-TSH-Rezeptor-Autoantikörpern und markiertem TSH durch an sich bekannte PEG-Fällung und anschließende Zentrifugation von der flüssigen Phase der Reaktionsmischung abtrennt.

5. Verfahren zur Bestimmung von Anti-TSH-Rezeptor-Autoantikörpern in einer humanen Serum- oder Plasmaprobe, bei dem man

- die zu bestimmenden Autoantikörper der Probe mit einem TSH-Präparat um die Bindungsstellen eines solubilisierten TSH-Rezeptors konkurrieren läßt, indem man eine Reaktionsmischung, die den TSH-Rezeptor, das TSH-Präparat sowie die Probe enthält, unter geeigneten Bedingungen inkubiert,
- den nicht an den TSH-Rezeptor gebundenen Anteil des TSH-Präparats an eine Festphase bindet und markiert, und
- anhand der Menge der an die feste Phase gebundenen Markierung auf die Menge der zu bestimmenden Autoantikörper in der Probe zurückrechnet,

dadurch gekennzeichnet, daß man

- als TSH-Rezeptor ein durch Extraktion und Solubilisierung aus einem humanen oder tierischen Schilddrüsenmaterial gewonnenes TSH-Rezeptor-Präparat oder einen solubilisierten rekombinanten TSH-Rezeptor verwendet,
- als TSH ein aus crudem bovinem TSH gewonnenen TSH-Präparats verwendet, das eine biologischen Aktivität von mehr als 40 IE TSH/mg Protein aufweist und erhältlich ist durch affinitätschromatographische Reinigung von handelsüblichem crudem bovinem TSH an einer anti-TSH-Antikörper-Säule und anschließende Ionenaustausch-Chromatographie an einem schwach sauren Kationenaustauscher mit endständigen Carboxylgruppen auf der Basis eines polyamidbeschichteten Kieselgels, und
- man zur Bereitung der Reaktionsmischung die Probe, das markierte TSH-Präparat und den TSH-Rezeptor im wesentlichen gleichzeitig zusammengibt und die so erhaltene Reaktionsmischung einer einstufigen Inkubation unterwirft.

6. Verfahren nach Anspruch 5, dadurch gekennzeichnet, daß man zur Bereitung der Reaktionsmischung für die Inkubation zuerst die Probe und das markierte TSH-Präparat zusammengibt und die Reaktion durch anschließende Zugabe des TSH-Rezeptors startet.

Claims

1. Method for the determination of anti-TSH receptor autoantibodies in a human serum or plasma sample, in which the autoantibodies to be determined in the sample are allowed to compete with a suitably labelled TSH preparation for the binding sites of a solubilized TSH receptor and the amount of autoantibodies to be determined is calculated from the amount of label bound to this TSH receptor, the

method comprising the steps

- incubation of a reaction mixture which contains the TSH receptor, labelled TSH and sample,
- conversion of the TSH receptor with the fractions of anti-TSH receptor autoantibodies and labelled TSH bound thereto into a solid phase and separation of the latter from the liquid phase, and
- measurement of the amount of labelled TSH bound in the solid phase by the physical or chemical detection method typical for the label used,

characterized in that

- the TSH receptor used is a TSH receptor preparation obtained by extraction and solubilization from a human or animal thyroid material or is a solubilized recombinant TSH receptor,
- the labelled TSH used is a radiolabelled TSH which was obtained by direct radioiodination of a TSH preparation which is obtained by purification of crude bovine TSH, has a biological activity of more than 40 IU TSH/mg protein and is obtainable by purification of commercial crude bovine TSH by affinity chromatography over an anti-TSH antibody column and subsequent ion exchange chromatography over a weakly acidic cation exchanger having terminal carboxyl groups and based on a polyamide-coated silica gel, and
- for the preparation of the reaction mixture, the sample, the labelled TSH preparation and TSH receptor are combined essentially simultaneously and the reaction mixture thus obtained is subjected to a one-stage incubation.

2. Method according to Claim 1, characterized in that, for the preparation of the reaction mixture, the sample and the labelled TSH preparation are first combined and the reaction is started by subsequent addition of the TSH receptor.

3. Method according to Claim 1, characterized in that a porcine TSH receptor preparation or a recombinant human TSH receptor is used.

4. Method according to any of the preceding Claims, characterized in that the TSH receptor with the fractions of anti-TSH receptor autoantibodies and labelled TSH bound thereto is separated from the liquid phase of the reaction mixture by PEG precipitation known per se and subsequent centrifuging.

5. Method for the determination of anti-TSH receptor autoantibodies in a human serum or plasma sample, in which

- the autoantibodies to be determined in the sample are allowed to compete with a TSH preparation for the binding sites of a solubilized TSH receptor by incubating, under suitable conditions, a reaction mixture which contains the TSH receptor, the TSH preparation and the sample,
- that fraction of the TSH preparation which is not bound to the TSH receptor is bound to a solid phase and labelled, and
- the amount of autoantibodies to be determined in the sample is calculated from the amount of label bound to the solid phase,

characterized in that

- the TSH receptor used is a TSH receptor preparation obtained by extraction and solubilization from a human or animal thyroid material or is a solubilized recombinant TSH receptor,
- the TSH used is a TSH preparation which is obtained from crude bovine TSH, has a biological activity of more than 40 IU TSH/mg protein and is obtainable by purification of commercial crude bovine TSH by affinity chromatography over an anti-TSH antibody column and subsequent ion exchange chromatography over a weakly acidic cation exchanger having terminal carboxyl groups and based on a polyamide-coated silica gel, and
- for the preparation of the reaction mixture, the sample, the labelled TSH preparation and the TSH receptor are combined essentially simultaneously and the reaction mixture thus obtained is subjected to a one-stage incubation.

6. Method according to Claim 5, characterized in that, for the preparation of the reaction mixture for the incubation, the sample and the labelled TSH preparation are first combined and the reaction is started by subsequent addition of the TSH receptor.

Revendications

1. Procédé pour la détermination d'auto-anticorps anti-récepteur de la TSH dans un échantillon de sérum ou de plasma humain, dans lequel on met en compétition les auto-anticorps à déterminer, de l'échantillon, avec une préparation de TSH marquée par un marqueur approprié, pour les sites de liaison d'un récepteur de la TSH solubilisé, et on calcule à l'aide de la quantité du marqueur lié à ce récepteur de la TSH, la quantité des auto-anticorps à déterminer, le procédé comportant les étapes consistant à:

- incuber un mélange de réaction qui contient le

English language translation of WO98/26

RECEPTOR BINDING ASSAY FOR DETECTING TSH RECEPTOR AUTOANTIBODIES

The invention relates to a competitive receptor binding assay for determining TSH receptor autoantibodies which occur in autoimmune disorders of the thyroid, especially in Graves' disease.

It is known that numerous disorders in which the thyroid is involved are autoimmune disorders in which there is production of autoantibodies against molecular structures of the thyroid which, in the context of the disorder, start to act as autoantigens. The most important known autoantigens of the thyroid are thyroglobulin (Tg), thyroid peroxidase (TPO) and, in particular, the TSH receptor (TSHr) (cf. Furmaniak J et al., Autoimmunity 1990, Vol. 7, p. 63-80).

The TSH receptor is a receptor which is located in the thyroid membrane and to which the hormone TSH (thyroid-stimulating hormone or thyrotropin), which is secreted by the pituitary, binds and thus induces secretion of the actual thyroid hormones, especially thyroxine. The TSH receptor belongs to the receptor family of G protein-coupled glycoprotein receptors with a large amino-terminal extracellular domain, to which the LH/CG receptor and the FSH receptor also belong. Elucidation of the chemical structure of the TSH receptor, that is to say of the sequence of the DNA coding therefor, and the amino acid sequence of the receptor itself, which can be deduced therefrom, succeeded at the end of 1989 (cf. Libert F. et al., Biochem. Biophys. Res. Commun. 165: 1250-1255; Nagayama Y. et al., Biochem. Biophys. Res. Commun. 165: 1184-1190; cf. also EP-A-0433509 or WO-A-91/09121; and WO-A-91/09137; WO-A-91/10735 and WO-A-91/03483; furthermore Yuji Nagayama & Basil Rapoport, in: Molecular Endocrinology, Vol. 6 No. 2, pp. 145-156 and the literature cited therein).

It is generally known that in the autoimmune thyroid disorder known as Graves' disease there is involvement of stimulating autoantibodies which are produced against the TSH receptor and interact with the latter in such a way that the thyroid is stimulated, which is manifested by hyperactivity of the thyroid (hyperthyroidism). Determination of autoantibodies against the TSH receptor thus has considerable clinical importance for diagnosing Graves' disease.

Apart from determination methods in which experimental animals or specific cell cultures are involved and which are now primarily of historic interest (cf. Schumm-Draeger et al., Akt. Endokr. Stoffw. 10 (1989), pp. 90-102), it has been possible to date to determine TSH receptor autoantibodies essentially in accordance with two methodological principles (cf. Morgenthaler N.G. et al., Exp Clin Endocrinol Diabetes 104 (1996) Suppl 4, pp.56-59):

In cell stimulation assays, the presence of stimulating TSH receptor autoantibodies, which are frequently referred to in the literature by the abbreviation TSI (TSI = thyroid stimulating immunoglobulins), is manifested by certain functions of suitable cells, which have natural or recombinant TSH receptors in their cell membrane and come into contact with an autoantibody-containing sample, being induced or enhanced by stimulation, in particular the production of cAMP (cyclic adenosine monophosphate). In these assays, which are also referred to as bioassays, there is selective measurement of the stimulating action, but the measurement is exceptionally complicated and therefore not very suitable for routine clinical diagnosis.

As an alternative to this, autoantibodies can also be determined by using competitive receptor binding assays, in particular radioreceptor assays, for example using the TRAK Assay® of B.R.A.H.M.S. Diagnostica GmbH. The procedure for determining TSH receptor autoantibodies in this conventional method is to allow the autoantibodies which are to be determined from a serum sample to

compete in liquid phase with a radiolabelled bovine TSH competitor for the binding sites of a detergent-solubilized porcine TSH receptor (cf. Southgate, K. et al., Clin. Endocrinol. (Oxford) 20, 539-541 (1984); Matsuba T. et al., J.Biochem. 118, pp. 265-270 (1995); EP 719 858 A2; Information on the TRAK Assay® product supplied by B.R.A.H.M.S Diagnostica GmbH). In order to determine the labelled TSH bound to the receptor preparation, after the incubation is complete the TSH receptor is removed from the liquid phase using a precipitating reagent and a subsequent centrifugation step. Determination of the receptor-bound labelled TSH takes place by measuring the radioactivity bound in the sediment. Since the determination is based on a competition between labelled TSH and the autoantibodies to be determined for common binding sites on the TSH receptor, the autoantibodies detected in this method are all those and only those which actually compete with TSH. Such competing autoantibodies able to inhibit TSH binding are also referred to in the literature as TBII (TBII = thyrotropin-binding inhibitory immunoglobulin) and the extent of their activity is also indicated as per cent so-called TBII activity.

It has been known for quite a long time now that in autoimmune disorders of the thyroid there is production of heterogeneous autoantibody populations which differ in composition. Moreover there is only partial identity of the stimulating autoantibodies and those which compete with TSH, that is to say there are stimulating autoantibodies which do not compete with TSH, and there are also autoantibodies which compete with TSH but have no stimulating action. In addition, there may also be autoantibodies present which neither have a stimulating action nor compete with TSH (cf., for example, Ludgate M et al., Mol. Cell. Endocrinol. 73 (1990), R13-R18; Filetti S et al., J. Clin. Endocrinol. Metab. 72; pp. 1096-1101, 1991; Morgenthaler N.G. et al., Exp Clin Endocrinol Diabetes 104 (1996) Suppl 4, pp. 56-59 and literature cited therein). The result of this is that

autoantibodies are detectable by radioreceptor assays in only about 80% to 90% of patients with Graves' disease (cf., for example, Rationelle Diagnostik in der Endokrinologie, Thieme Verlag, p. 49, Section: TSH-Rezeptor-autoantikörper (TSH-RAK); or else Ropars A. et al., Cell. Immunol. 161, pp.262-269 (1995); Ohmori M. et al., Biochem. Biophys. Res. Commun. 174, No. 1 (1991), pp. 399-403; Endo T. et al., Biochem. Biophys. Res. Commun. 181, No. 3 (1991), pp. 1035-1041; Gupta MK, Clin. Biochem. 25, pp. 193-199 (1992)). Since nondetection of some of the autoantibodies occurring in Graves' disease is causally linked to the principle of measurement of competitive radioreceptor assays to date, it has already been proposed to carry out, despite the considerable effort, a supplementary bioassay measurement to determine stimulating TSI autoantibodies if there is an evident discrepancy between the clinical picture of a patient with Graves' disease and the result of measurement to determine competing TBII autoantibodies (Derwahl M. et al., Exp. Clin. Endocrinol. 100 (1992), pp. 75-79).

Apart from the limited clinical value which has been described, competitive radioreceptor assays known to date for detecting TSH receptor autoantibodies have fundamental disadvantages of a practical nature which can be ascribed to the fact that the binding ability of TSH receptor preparations generally reacts very sensitively to changes in the receptor or in the biomolecule bound by it. The binding of biomolecules which are peptides or proteins in nature, for example hormones or else autoantibodies, to receptors is as a rule very complicated in nature, and the formation of a specific binding between receptor and biomolecule is very much more sensitive to structural alterations, in particular of the receptor, than is the case with a usual antigen/antibody binding pair which is the basis of most immunoassays in which receptors are not involved. Attempts to immobilize and/or to label the TSH receptor have as a rule led to structural alterations which greatly impair the functionality of the receptor. The result of this is that

any basic types of assay which are available utilizing antibody/antigen binding in the case of immunoassays, especially those which use immobilized binding partners and in which there is direct determination, at the end of the measurement, of the concentration of a label bound to a solid phase, or in which bulky molecules such as enzymes, enzyme subtrates or chemoluminescent labels are used for labelling, have not hitherto been usable for carrying out receptor binding assays for determining TSH receptor autoantibodies in practice. Since the measurement of a label bound to a solid phase is the basis for most automated assays for serial measurements, it has not to date been possible to carry out the known assays for determining TSH receptor autoantibodies with such automation.

DE Patent 43 28 070 C1 describes a type of receptor binding assay which operates by the coated tube technique and with which the difficulty of preparing labelled or immobilized functional receptor preparations is avoided by binding to the solid phase the constituents of a competing reaction system which to a certain extent represents a "shadow" of the actual receptor binding reaction. However, the disclosed methodological principle has proved to be too complicated, and therefore of little practical use, for designing assays for routine clinical diagnosis. Express reference is additionally made to the general statements in said patent about the problems of receptor binding assays in general and of those for determining TSH receptor autoantibodies in particular.

EP-B-0 488 170 furthermore discloses cell-free receptor binding assays in which recombinant fusion receptors consisting of an amino-terminal receptor protein and a carrier protein, in particular the constant part (Fc) of the heavy chain of an immunoglobulin, are employed and are coupled by means of an antiserum or of a monoclonal antibody to a solid phase. The suggested receptors do not belong to the class of high molecular weight G protein-coupled glycoprotein receptors. Furthermore, immobilization by binding a carrier protein which

is the Fc part of an immunoglobulin is not very suitable for receptor binding assays intended for the determination of autoantibodies, because the autoantibodies themselves are immunoglobulins and may bind to the immobilization system.

DE 41 20 412 C1 furthermore discloses a method for determining autoantibodies which, for the determination, makes use of the perturbation of a sandwich composed of an immobilized specific antibody, of a crude autoantigen in the form of a suitable organ extract, in particular TPO, and of another, labelled, antibody by the autoantibodies against the autoantigen which are present in the sample. Autoantibodies present in a sample can for the purpose of perturbation interact in principle with every single or any two immunological binding(s) involved in the structure of the complete sandwich. It has not to date been possible, because of the described sensitivity of receptors and the lack of autoantibodies with the required selectivity, to apply this methodological principle to cases where the autoantigen is a receptor and the labelled binding partner is a labelled hormone such as, for example, TSH.

After the molecular structure of the TSH receptor was elucidated, numerous research groups produced monoclonal and polyclonal antibodies against human recombinant TSH receptors, against the N-terminal extracellular part of such receptors (containing 398 amino acids excluding the signal peptide) and against conjugates of shorter receptor peptide part sequences (cf., for example, N.G. Morgenthaler et al., Exp Clin Endocrinol Diabetes 104 (1996) Suppl. 4, pp.56-59; Seetharamaiah GS et al., Endocrinology 134, No. 2, pp. 549-554 (1994); Desai RK et al., J. Clin. Endocrinol. Metab. 77:658-663, 1993; Dallas JS et al., Endocrinology 134, No. 3, pp. 1437-1445 (1994); Johnstone AP et al., Mol. Cell. Endocrinol. 105 (1994), R1-R9; Seetharamaiah GS et al., Endocrinology 136, No. 7, pp. 2817-2824 (1995); Nicholson LB et al., J. Mol. Endocrinol. (1996) 16, pp. 159-170; Ropars A et al., Cell. Immunol. 161, pp.262-269 (1995);

Ohmori M et al., Biochem. Biophys. Res. Commun. 174, No. 1 (1991), pp. 399-403; Endo T. et al., Biochem. Biophys. Res. Commun. 181, No. 3 (1991), pp.1035-1041; Costagliola S. et al., Endocrinology 128, No. 3, pp.1555-1562, 1991; Marion S. et al., Endocrinology 130, No. 2, pp.967-975 (1992); J. Sanders et al., J. Endocrinol. Invest. 19 (Suppl. No. 6); 1996 and other publications cited in said references), with the aim of elucidating the epitopes of the TSH receptor which are responsible for the TSH binding and the antibody binding. The various antibodies have been tested for their ability to bind with the TSH receptor or part sequences thereof produced by recombination and, in particular, for their ability to interfere with the binding of TSH to various forms or fragments of the TSH receptor. Since the various polyclonal or monoclonal antibodies were produced by immunizing various animals and/or using recombinant material from various expression systems and, in addition, TSH receptors of various origins produced by recombination, or partial peptides thereof, were often used in the binding assays, and since it furthermore emerged that glycosylation and/or correct folding of the receptor peptides was likely to be crucial for the binding of many antibodies, the epitope structure of native TSH receptors and the epitope-specific binding behaviour of the autoantibodies occurring in polyclonal autoantibody populations have not yet been completely elucidated.

In the publication by Dallas JS et al., Endocrinology 134, No. 3, pp. 1437-1445 (1994), for example, there is use of a partial recombinant TSH receptor which was produced with the aid of the baculovirus/insect cell expression system and which has the amino acids of the extracellular domain of the human TSH receptor without the N-terminal signal peptide to immunize rabbits, and specific antibody fractions were obtained, by affinity chromatography using synthetic peptides each of about 20 amino acids, from the immunoglobulin fractions produced. These antibody fractions were then investigated inter alia for their suit-

ability for blocking, in a commercial receptor binding assay, the binding of TSH to a solubilized porcine TSH receptor. The antibodies showed no stimulatory activity.

In the publication by Seetharamaiah GS et al., Endocrinology 136, No. 7, pp. 2817-2824 (1995) there is a description of how the same partial recombinant TSH receptor as in the preceding publication was employed for immunizing mice and for producing monoclonal antibodies against individual epitopes of the TSH receptor by standard techniques. A similar procedure is described in Nicholson LB et al., J. Mol. Endocrinol. (1996) 16, pp. 159-170.

According to Seetharamaiah GS et al., Endocrinology 134, No. 2, pp. 549-554 (1994), a partial recombinant TSH receptor produced as above is subsequently folded and then tested for its suitability for binding radiolabelled TSH. For this, it is reacted in liquid phase with radiolabelled TSH. The resulting complex is then removed as quantitatively as possible from the reaction mixture by adding, as part of a precipitation system, an antibody which has been produced by immunizing rabbits with a conjugate of a partial peptide which contains amino acids 357 to 372 of the complete TSH receptor sequence and which has been found not to inhibit the binding of TSH to the unfolded partial recombinant TSH receptor (Desai RK et al., J. Clin. Endocrinol. Metab. 77:658-663, 1993). The added antibody, and the complexes containing it and bound radiolabelled TSH, are then precipitated using protein A, which binds non-specifically to every antibody. Under the experimental conditions, it appears that the binding of protein A to the receptor-bound antibody does not impair simultaneous TSH binding.

The information obtained with antibodies against recombinant TSH receptors or parts thereof led to the proposal to use, for determining TSH receptor autoantibodies, a third methodological principle, which is known per se, in the form of an immunoprecipitation assay, in which the reagent used for precipitation is a preparation

of an extracellular part of a recombinant TSH receptor labelled by incorporation of ^{35}S-labelled methionine. In an assay of this type there is no selectivity either for TSI or for TBII. (N.G.Morgenthaler et al., Exp Clin Endocrinol Diabetes 104 (1996) Suppl. 4, pp.56-59). However, preparation of the labelled receptor by in vitro translation is extremely complicated and costly, and there is no measuring equipment suitable for routine clinical measurement of the radiation emitted by ^{35}S. The method is therefore unsuitable as method of measurement for routine clinical diagnosis.

It is an object of the present invention to design a competitive receptor binding assay for determining TSH receptor autoantibodies which does not have the described disadvantages of such competitive receptor binding assays of the prior art.

A particular object of the present invention is to design an improved competitive receptor binding assay for determining TSH receptor autoantibodies with which it is possible to immobilize the TSH receptor complexes, formed from the reactants in the determination method, for measurement on a conventional solid phase, and to overcome the existing restrictions concerning the labels which can be used, so that such receptor binding assays can also be carried out automatically.

A further particular object of the present invention is to design such improved competitive receptor binding assays for detecting TSH receptor autoantibodies so that the clinical value is increased by comparison with such assays of the prior art.

It is a further object of the present invention to design reagent kits necessary for carrying out such improved receptor binding assays in routine clinical diagnosis.

The said objects are achieved for a competitive receptor binding assay according to the precharacterizing clause of Claim 1 at least in part by radioreceptor assays which have the features specified in the characterizing part of Claim 1.

Advantageous developments of the improved receptor binding assays according to the invention are evident from the dependent claims, in particular in conjunction with the explanations hereinafter in the present description.

The object of designing an appropriate reagent kit is achieved by a reagent kit for carrying out a method according to the invention, which contains at least one in each case of constituents (i), (ii) and (iii) according to Claim 15.

In order to cover various possible embodiments of the receptor binding assay of the present invention, Claim 1 uses generalizing words both for describing known features of such receptor binding assays and for characterizing those features by which the receptor binding assay according to the invention differs from one in the prior art, and these words are introduced and explained below:

As explained in the introductory part, the known competitive receptor binding assays, all of which are designed as radioreceptor assays, contain the following assay constituents:

A solubilized TSH receptor (i), in particular a solubilized native animal (porcine) TSH receptor obtained from animal thyroid membranes.

The known radioreceptor assays furthermore contain radiolabelled bovine TSH (ii) which competes with TSH receptor autoantibodies from a serum sample (TBII) for common binding sites on the TSH receptor used. Since this is the competition which has hitherto been of prime importance and moreover makes it possible to detect most of the autoantibodies occurring, namely 80 to 90%, the labelled TSH of the prior art is covered by the definition of "primary competitor" used in Claim 1. However, this definition also embraces other forms of "primary competitors" whose use has been made possible for the first time by the present invention. Thus, it is also possible to use labelled selective antibodies against the TSH receptor, which are described in detail hereinafter,

as competitors for TBII or else, where appropriate, other autoantibody fractions. It is furthermore intended that a TSH bound to a solid phase be able, because of the competition with TBII, to comply with the definition of "primary competitor".

As likewise explained at the outset, in the prior art the complexes of the TSH receptor and constituents, bound thereto, of the reaction mixture, that is say labelled TSH and autoantibodies, are precipitated from the reaction mixture using a precipitating reagent and are removed therefrom by centrifugation. The precipitating reagent is to be regarded as means according to (iii) within the meaning of the precharacterizing clause of Claim 1. In a competitive receptor binding assay according to the invention, a means (iii) of this type corresponds to a reactant which is bound to a solid phase and which is primarily a specific TSH receptor antibody which is immobilized on a solid phase but which can also be, in a "mirror image" embodiment, a TSH bound to a solid phase.

It is a realization which is essential for the present invention that it is possible with the aid of immobilized selective antibodies which are directed specifically against a suitable part sequence of the TSH receptor to carry out an immobilization also of a native TSH receptor (that is to say a human, animal or else recombinant TSH receptor whose folding/glycosylation at least essentially corresponds to that of a naturally occurring TSH receptor) from solubilized TSH receptor preparations of any type, with negligible impairment of the ability of the TSH receptor to bind to TBII autoantibodies and labelled TSH. This realization thus created the precondition for carrying out a receptor binding assay for determining TSH receptor autoantibodies in such a way that determination of the amount of the bound labelled competitor, for example the primary competitor radioiodine-labelled TSH, can take place in a form bound to a solid phase. However, it has further emerged, surprisingly, that it is possible by immobilizing the TSH

receptor on a solid phase with the aid of selective TSH receptor autoantibodies to improve considerably the clinical value of competitive receptor binding assays for determining TSH receptor autoantibodies. This is because, as will be explained in more detail below, it has emerged that with a suitable choice of the specific antibody bound to the solid phase the determination system contains at least one other binding site for which autoantibodies from the sample can compete.

If the antibody bound to a solid phase is chosen so that it acts as "secondary competitor" which competes with other autoantibodies in the sample as the TBII detected by competition with the "primary competitor" there is an increase in the clinical value. This is because there is production of a measurement system which can be regarded as analogous to the determination system depicted diagrammatically in Fig. 3 in DE 41 20 412, in which there is double perturbation at different binding sites of a sandwich due to heterogeneous polyclonal autoantibodies from a patient's sample. Since such a perturbation is sufficient to prevent binding of the label to the solid phase, the measurement range is extended and the sensitivity is increased. The said variant using two antibodies and a crude antigen is also embraced by US Patent 5,501,955.

It has now emerged, surprisingly, that with the method according to the invention using, for example, a specific antibody which has been produced against a synthetic peptide with amino acids 20 to 39 of the amino acid sequence of the complete TSH receptor (including signal peptide) all patients classified on the basis of signs and symptoms as patients with Graves' disease were found to be autoantibody-positive although only 80% had been found to be positive with the conventional radio-receptor assay. It is thus possible by using a "secondary competitor" which competes with autoantibodies other than TBII which occur in Graves' disease to increase considerably the clinical value of the receptor binding assay for diagnosing Graves' disease.

The "secondary competitor" in this case preferably competes with autoantibodies (TSI autoantibodies) which have been found to be stimulating in a cell stimulation assay but which are not detected by the conventional radioreceptor assay through lack of competition with TSH. However, it is unnecessary for the autoantibodies with which the "secondary competitor" competes in fact to have a stimulating action and thus intensify the signs and symptoms of Graves' disease. The autoantibodies with which the "secondary competitor" competes can also belong to an autoantibody subpopulation which has a blocking action or is unimportant for the actual pathological event. As long as such an autoantibody subpopulation occurs always or frequently in the serum of patients with Graves' disease, the clinical value of the receptor binding assay will be increased by comparison with the conventional radioreceptor assay which detects only TBII autoantibodies.

Thus, particularly suitable selective antibodies which form preferred "secondary competitors" are those directed against sections of the TSH receptor which are not involved in the TSH binding but against which autoantibodies are produced in Graves' disease. Investigations to date using polyclonal antibodies purified by affinity chromatography or antibodies produced as monoclonal antibodies have led to some sections of the TSH receptor being considered as particularly suitable binding regions for such selective antibodies. However, when interpreting the literature data to date, it must be taken into account that most selective antibodies were produced against TSH receptor peptides produced by recombination or synthetic partial peptides and therefore probably are sequential in nature. Furthermore, most of the antibodies described in nature prove to be without effect in the cell stimulation assay. This indicates that, despite competition with autoantibodies from sera, it is not possible to equate the artificially produced antibodies with the autoantibodies to be determined. The binding behaviour of the artificially produced antibodies

to native TSH receptor preparations may furthermore differ because of the folding of the native TSH receptor and its glycosylation from the binding behaviour to synthetic peptides or TSH receptor fragments produced by recombination. However, with knowledge of the present invention it is possible in the individual case to establish even in relatively simple routine tests the suitability of particular specific antibodies for the method according to the invention and their ability to act as "secondary competitor".

The tests described hereinafter were carried out using an immobilized specific antibody which is known to bind to a peptide from the region of amino acids 20 to 39 of the complete TSH receptor, and labelled bovine TSH as conventional tracer ("primary competitor"). The antibody used was a monoclonal antibody which had been selected and produced by the conventional hybridoma technique after immunization with the aid of a conjugate of the appropriate synthetic peptide. There have been descriptions in the literature of various polyclonal or monoclonal specific antibodies which likewise bind to the same region or overlapping regions of the amino-terminal end of the extracellular domain of the TSH receptor (cf. Dallas J.S. et al., Endocrinology 134, No. 3, pp. 1437-1445 (1994); Seetharamaiah G.S. et al., Endocrinology 136, No. 7, pp. 2817-2824 (1995); Nicholson LB et al., J. Mol. Endocrinol. (1996) 16 pp. 259-170; Ohmori, M. et al., J. Endocrinol. (1992) 135, pp. 479-484; Endo, T. et al., Biochem. Biophys. Res. Commun. 177, pp. 145-150 (1991); Hirooka, Y. et al., Med. Sci. Res. 1992, 20, pp. 639-640). The production techniques and antibody types described in the references mentioned are suitable in principle as replacement for the antibody specifically used in the tests, although it should be noted that it is known that antibodies against sequences of the TSH receptor which are close neighbours may belong to entirely different antibody types.

Suitable alternatives to the antibody used in the Examples, which is directed against amino acids 20 to 39

of the human TSH receptor, are also antibodies produced, for example, against the regions 287 to 301, 361 to 380 or 739 to 758. The specific properties must, however, be checked in the individual case on the basis of the tests described hereinafter.

If the difference from the original design of the conventional radioreceptor assay is even greater and another labelled selective antibody is used in place of radiolabelled TSH, the above distinction between "primary competitor" and "secondary competitor" may become less clear or even lose all meaning. This changes nothing in the principle of the receptor binding assay according to the invention or the fact that such assays are also within the scope of the present invention.

The fact that the method according to the invention makes it possible to immobilize the TSH receptor complex formed in the measurement solution with a label bound directly thereto or a label introduced after the immobilization, and that furthermore reagents with labels other than radionuclides can be used, means that practical manipulation of TSH receptor autoantibody determination methods can be adapted very much better than hitherto possible to the requirements of clinical practice. The incubation schemes can also be varied, and the incubation scheme described in the following Examples does not appear to be an absolute necessity.

Solid phases which can be employed are plastic surfaces, microparticles, magnetic particles, filters, polymer gel materials and other known solid phase supports. The possibility of using labelled autoantibodies and immobilized TSH (concerning the ability of TSH receptors to bind to immobilized TSH, cf. Leedman, P.J. et al., J. Clin. Endocrinol. Metab. 69; pp. 134-141, 1989) considerably increases the range of application of the assay, especially also with regard to the choice of suitable direct or indirect labels. The method according to the invention also makes TSH receptor autoantibody assays automatable. It is thus possible to adapt the assay design so that it can be carried out in known

automated systems (cf., for example, Elecsys system of Boehringer Mannheim or ACS 180 system of Ciba Corning). With such automated systems, the automatic operation involves pipetting the samples, and it is then possible to admix magnetic particles which are coated with a suitable specific antibody against the TSH receptor or with TSH, mixed with labelled TSH or any suitable labelled antibody (for example labelled with ruthenium complex or acridinium ester), and finally in principle any suitable TSH receptor material is added. After the incubation, the usual solid/liquid separation is possible, and then the signal can be determined, where appropriate after inducing a signal by adding suitable reagents. The sequence of pipetting steps indicated by way of example is moreover variable.

The method according to the invention is explained below in more detail by means of a specific example with reference to two Figures.

These show:

Fig. 1 a standard plot, obtained in a usual way, for the embodiment of the method according to the invention described in the example hereinafter; and

Fig. 2 a comparison of the results of measurement on a group of patients with Graves' disease, obtained with the method according to the invention and with the conventional radioreceptor assay (TRAK assay® supplied by B.R.A.H.M.S Diagnostica GmbH).

Description of the tests

Materials:

Various commercially available materials were used in the tests described hereinafter, specifically components of the TRAK assay® supplied by B.R.A.H.M.S Diagnostica GmbH, an NAP25 desalting column supplied by Pharmacia for purifying the antibody prepared for the immobilization, Carbolink material supplied by Pierce as solid phase support material, and sodium periodate

supplied by Fluka.

1. Production of monoclonal antibodies against the TSH receptor

Various monoclonal antibodies against selected peptides of the hTSH receptor were produced by prior art methods (see above). A monoclonal antibody directed against a peptide which corresponds to amino acids 20 to 39 of the complete human TSH receptor (amino acid sequence GGMGCSSPBCECHQEEDFRV) was used for the further investigations.

2. Production of monoclonal antibodies bound to a solid phase

250 mg of periodate are dissolved in 20 ml of an ascites solution which is diluted 1:10 with PBS (phosphate-buffered saline; 50 mM sodium phosphate, 100 mM NaCl, pH 7.5) and which contains the abovementioned monoclonal antibody, and incubated at room temperature for 30 min. The reaction solution is desalted through an NAP25 desalting column (Pharmacia) in accordance with the manufacturer's instructions. The desalted protein is mixed with Carbolink material (10 ml) washed in PBS.

After incubation at 4°C with constant shaking overnight, 0.5 ml of the resulting gel with the monoclonal antibody bound thereto was introduced into plastic columns (0.5 × 4 cm) and washed with 25 mM sodium citrate buffer, pH 2.5 (washing volume 2 ml). The columns were equilibrated in PBS (2 ml) and then used for the further investigations.

3. A radioreceptor assay was carried out using the columns described and using components of the TRAK assay® (B.R.A.H.M.S Diagnostica GmbH)

The radioreceptor assay was carried out (construction of standard plots, measurement of patients' samples) in accordance with the instructions for carrying out the TRAK assay®. The following pipetting scheme was used for this:

50 µl of the sample are pipetted.

Then 50 µl of solubilized porcine TSH receptor are

added by pipette.
The mixture is incubated at room temperature for 15 min.
100 µl of tracer (radioiodinated TSH) are pipetted in.
The resulting reaction mixture is transferred to the solid phase (column with Carbolink material) loaded with the monoclonal antibody as above.
The reaction mixture is incubated at room temperature for one hour.
The column is washed with 2 ml of PBS.
The radioactivity bound to the solid phase is measured in a gamma counter, either using the column with solid phase directly or after elution of the bound proteins with 1 ml of 25 mM sodium citrate buffer, pH 2.5.
The procedure for constructing a standard plot for evaluating the results of measurement of patients' sera corresponded, using the standards from the commercial TRAK assay® supplemented by a standard which contains 2000 U TSH/ml.

Results:

Using the standards, a standard plot (Fig. 1) corresponding to that of a usual TRAK assay® was obtained, that is to say increasing concentrations of added unlabelled TSH led to a reduction in the bound radioactivity.

When serum samples from patients with Graves' disease were measured in the commercial TRAK assay® and by the method according to the invention, the following results were obtained:

Sera from 45 healthy control subjects and from 39 patients with Graves' disease were measured using the conventional TRAK assay® and by the method according to the invention. The results of the measurements are compiled in Table 1.

The sera from the patients with Graves' disease are derived from samples before the first therapy or

within six weeks after starting therapeutic measures.

Table 1

	Control Group (U/1)					Graves' disease (U/1)			
No.	TRAK assay		method of invention			TRAK assay		method of invention	
1	p	12.3	p	16.4	1	p	42.3	p	31.3
2	n	1.2	n	6.4	2	n	3	p	27.7
3	n	7.2	n	13.7	3	n	6.1	p	44.2
4	n	3.8	n	7.9	4	p	15.5	p	41.5
5	n	7.2	n	4.7	5	p	936	p	966
6	n	1.7	n	6.5	6	p	15.5	p	47.4
7	n	3.4	n	9.7	7	p	108.3	p	164.8
8	n	4.4	n	1.1	8	p	16.4	p	26.7
9	n	3.8	n	1.2	9	p	26.3	p	84.7
10	n	2.5	n	12.4	10	n	9.4	p	58.9
11	n	4.7	n	7.8	11	p	56.3	p	239.2
12	n	2	n	2.3	12	n	3.4	p	17.3
13	n	10.6	n	1.3	13	p	19.5	p	34.1
14	n	1.1	n	1.4	14	p	13.2	p	229.1
15	n	3.9	n	1.5	15	p	40.3	p	29.8
16	n	2.1	n	1.6	16	p	15	p	25
17	n	2.5	n	1.7	17	p	23.6	p	271.7
18	n	3	n	1.8	18	p	18.6	p	46.6
19	n	4.8	n	1.9	19	p	697	p	671
20	n	1.8	n	2	20	p	60.8	p	150.6
21	n	3.1	n	2.1	21	p	86.5	p	133.7
22	n	9.3	n	2.2	22	p	19.3	p	27.3
23	p	16.6	p	15.5	23	p	26.8	p	47.5
24	n	2.1	n	2.3	24	p	61.4	p	255.9
25	n	4.9	n	10.2	25	p	49.3	p	135.6
26	n	4.9	n	2.5	26	p	149	p	439.5
27	n	5	n	12	27	p	16.7	p	28.6
28	n	1.8	n	2.6	28	p	176	p	303.2
29	n	2.9	n	2.7	29	n	1.1	p	14.9
30	n	2.6	n	2.8	30	n	1.2	p	17.1
31	n	7.9	n	2.8	31	p	182.3	p	644
32	n	4	n	2.9	32	p	23.7	p	40.5
33	n	2.5	n	13.6	33	p	17.8	p	23
34	n	1.9	n	2.9	34	n	1.3	p	15.6
35	n	1.8	n	10.1	35	p	95.1	p	77.8

36	n	2.3	n	5.2	**36**	p	19.8	p	36
37	n	1.9	n	1	**37**	p	13.7	p	165
38	n	2.3	n	1.1	**38**	p	12.8	p	29.9
39	n	5.7	n	2.9	**39**	n	9.8	p	14.7
40	n	6.4	n	1.2					
41	n	1.6	n	5.9					
42	n	1.6	n	1	**cut off**		**11.0**		**14.0**
43	n	1.5	n	1.9					
44	n	4	n	5					
45	n	1.4	n	4					

p = TSH receptor autoantibody-positive

n = TSH receptor autoantibody-negative

Mean		**4.09**	**4.88**
1 s		**3.18**	**4.40**

The mean for the measurement of the autoantibody-free sera in the control group (n=45) is 4.1 ± 6.4 (2s) U/ml for the TRAK assay® and 4.8 ± 8.8 (2s) U/ml with the method according to the invention.

In order to maintain a comparable specificity of 95.5% with the two assays, the cut-off (boundary line between values for negative and positive samples) was set at 11 U/ml for the conventional method and at 14 U/ml for the method according to the invention. Using these cut-off values, 79.5% of the Graves' patients (31 of 39) were detected as positive in the established TRAK assay® radioreceptor method.

Measurement of the same group of patients by the method according to the invention surprisingly showed that 100% of the Graves' patients (39 of 39) were auto-antibody-positive. The samples found to be negative in the conventional TRAK assay® (samples No. 2, 3, 12, 29, 30, 34, 39) were detected unambiguously as positive by the method according to the invention.

In addition, a further advantage which emerged for the method according to the invention was that most of the samples were found to have a significantly stronger measurement signal. The results obtained are shown in a graph in Fig. 2 for clarity.

The additional possibility which exists with the

method according to the invention for interfering with the interaction between specific antibody bound to a solid phase and the TSH receptor complex with radio-labelled TSH bound thereto by autoantibodies present in the sample not only increases the clinical value of the assay for diagnosing Graves' disease but, in addition, also produces a considerably stronger and thus more reliable measurement signal by the method according to the invention for patients identified as positive in both assays. The clinical value of determining TSH receptor autoantibodies whose occurrence is characteristic of Graves' disease is thus considerably increased.

Claims

1. Competitive receptor binding assay for determining TSH receptor autoantibodies in a biological sample, in which the sample is reacted in a reaction mixture simultaneously or successively with (i) a TSH receptor in the form of a solubilized native human or animal or recombinant TSH receptor preparation, (ii) a primary competitor for at least some of the TSH receptor autoantibodies to be expected in the sample and (iii) a means for removing a complex of the TSH receptor and constituents, bound thereto, of the reaction mixture from the liquid phase, and in which the presence and/or amount of TSH receptor autoantibodies to be determined in the biological sample is found on the basis of the amount of a competitor in the TSH receptor complex removed from the liquid phase or on the basis of the remaining amount of unbound primary competitor in the liquid phase, for which purpose the competitor is employed in labelled form or is labelled after the solid/liquid separation, characterized in that the reaction is furthermore carried out in the presence of at least one monoclonal or polyclonal antibody which is specifically directed against a peptide partial sequence of the TSH receptor, and in that this specific antibody is employed as means for immobilizing a complex of TSH receptor and primary competitor and/or as secondary competitor for a further part of the TSH receptor autoantibodies to be expected in a sample, where the primary or the secondary competitor is labelled or can be selectively labelled.

2. Receptor binding assay according to Claim 1, characterized in that the at least one specific antibody is employed in a form bound with solid phase, and the primary competitor is labelled TSH or another, labelled, TSH receptor antibody, where the binding of the specific antibody bound to a solid phase is essentially unaffected by the binding of the primary competitor to the TSH receptor.

3. Receptor binding assay according to Claim 2,

characterized in that the antibody bound to a solid phase is simultaneously a secondary competitor which competes with those TSH receptor autoantibodies from the sample which do not impede the binding of the primary competitor to the TSH receptor for an epitope which is not recognised by the primary competitor.

4. Receptor binding assay according to Claim 2, characterized in that the other, labelled, TSH receptor antibody competes with TSH for epitopes of the TSH receptor.

5. Receptor binding assay according to Claim 3, characterized in that the antibody bound to a solid phase competes as secondary competitor with those TSH receptor autoantibodies from the sample whose occurrence is characteristic of Graves' disease but which do not compete with TSH.

6. Receptor binding assay according to Claim 5, characterized in that the TSH receptor autoantibodies competing with the secondary competitor belong to the stimulating autoantibodies (TSI).

7. Receptor binding assay according to Claim 1, characterized in that the at least one antibody in labelled form is employed as secondary competitor in the liquid phase, and in that a TSH bound to a solid phase is employed as means for removing a complex of the TSH receptor and constituents of the reaction mixture bound thereto, and as primary competitor.

8. Receptor binding assay according to any of Claims 1 to 7, characterized in that the at least one other antibody has been produced by immunization known per se of a suitable animal with a recombinant partial TSH receptor, in particular the extracellular domain or an extracellular loop of the membrane-anchored domain of a TSH receptor, and by fractionation by affinity chromatography of the antibodies formed, using immobilized partial peptides of the partial TSH receptor used for the immunization.

9. Receptor binding assay according to any of Claims 1 to 7, characterized in that the at least one other

antibody is a monoclonal antibody which has been produced by immunization known per se of a suitable animal with a recombinant partial TSH receptor, in particular the recombinant extracellular part of a TSH receptor or a shorter partial peptide thereof, fusion known per se of antibody-producing spleen cells with myeloma cells and selection known per se of individual antibody-producing hybrid cells and cultivation thereof.

10. Receptor binding assay according to either of Claims 8 or 9, characterized in that the at least one other antibody was produced against a TSH receptor partial peptide which is selected from peptides with amino acids 20-39, 32-54, 287-301, 361-381 or 739-758 of the amino acid sequence of the complete TSH receptor.

11. Receptor binding assay according to any of Claims 1 to 10, characterized in that the solid phase used is a support in the form of particles, test tubes or microtitre plates made of plastic or glass or in the form of magnetic polymer particles, polymer gels or filters, and the at least one antibody or TSH is employed bound directly or indirectly to such a support.

12. Receptor binding assay according to any of Claims 1 to 11, characterized in that a label known per se, which is selected from a radionuclide, an enzyme, an enzyme subtrate or a constituent of a chemiluminescence or fluorescence labelling system, is used for labelling the primary or secondary competitor.

13. Receptor binding assay according to any of Claims 1 to 11, characterized in that a primary or secondary competitor which is bound to a component of a specific binding system is employed, and in that the TSH receptor complex formed is labelled or immobilized by reaction with a reactant which has the second component, which is bound to a detectable label or a solid phase, of the specific binding system.

14. Receptor binding assay according to any of Claims 1 to 13, characterized in that the TSH receptor autoantibodies to be determined are receptor-stimulating autoantibodies whose occurrence in a human serum is charac-

teristic of Graves' disease.

15. Reagent kit for carrying out a competitive receptor binding assay according to any of Claims 1 to 14, characterized in that it contains, besides other conventional components of a reagent kit of this type:

(i) a solubilized TSH receptor preparation,

(ii) labelled TSH or a labelled specific TSH receptor antibody, and

(iii) a solid phase support to which a specific TSH receptor antibody or TSH is bound.

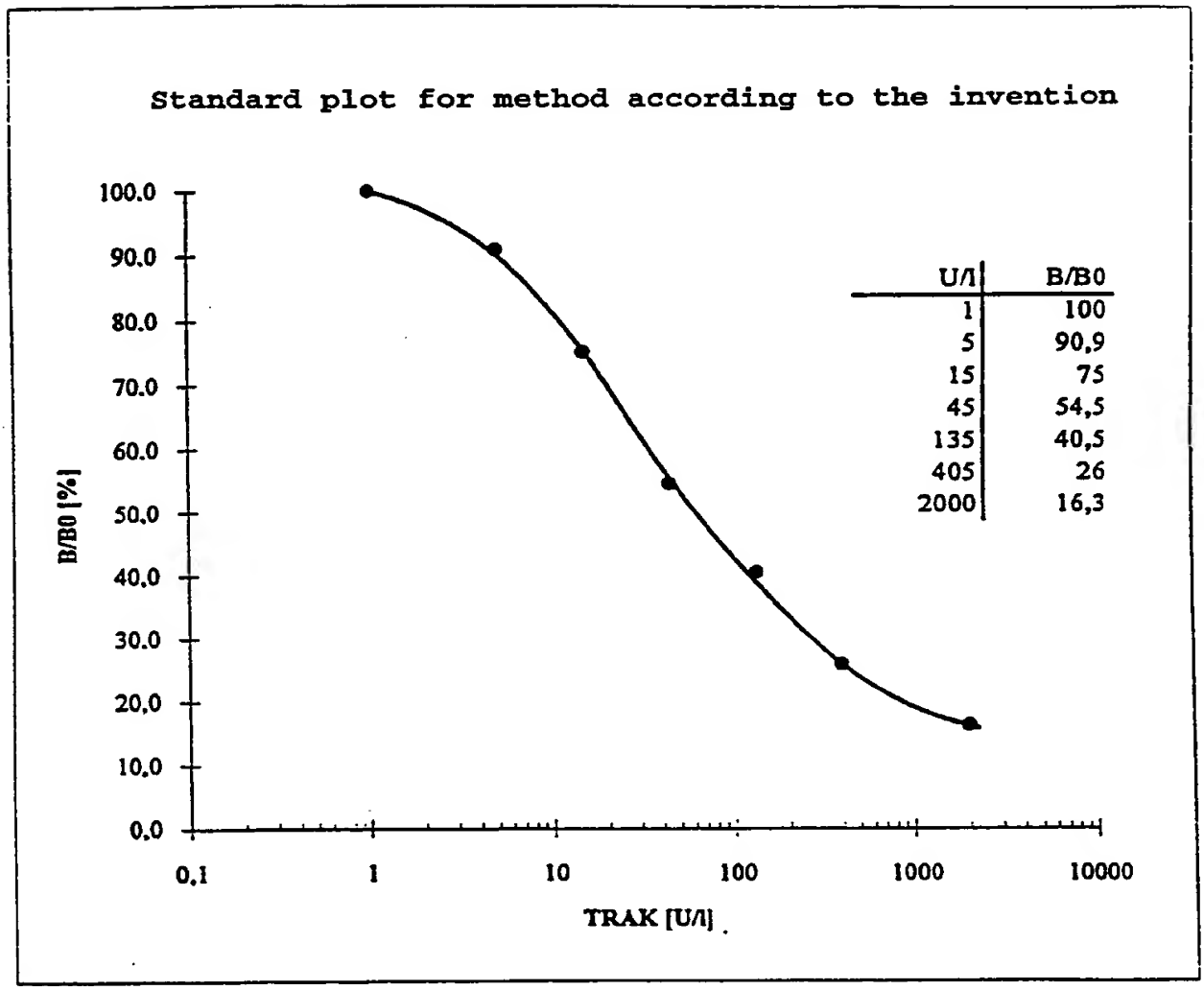
Standard plot for method according to the invention
B/B0 [%]
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0.1
1
10
100
1000
10000
TRAK [U/l]
U/l
B/B0
1
100
5
90,9
15
75
45
54,5
135
40,5
405
26
2000
16,3

Fig. 1

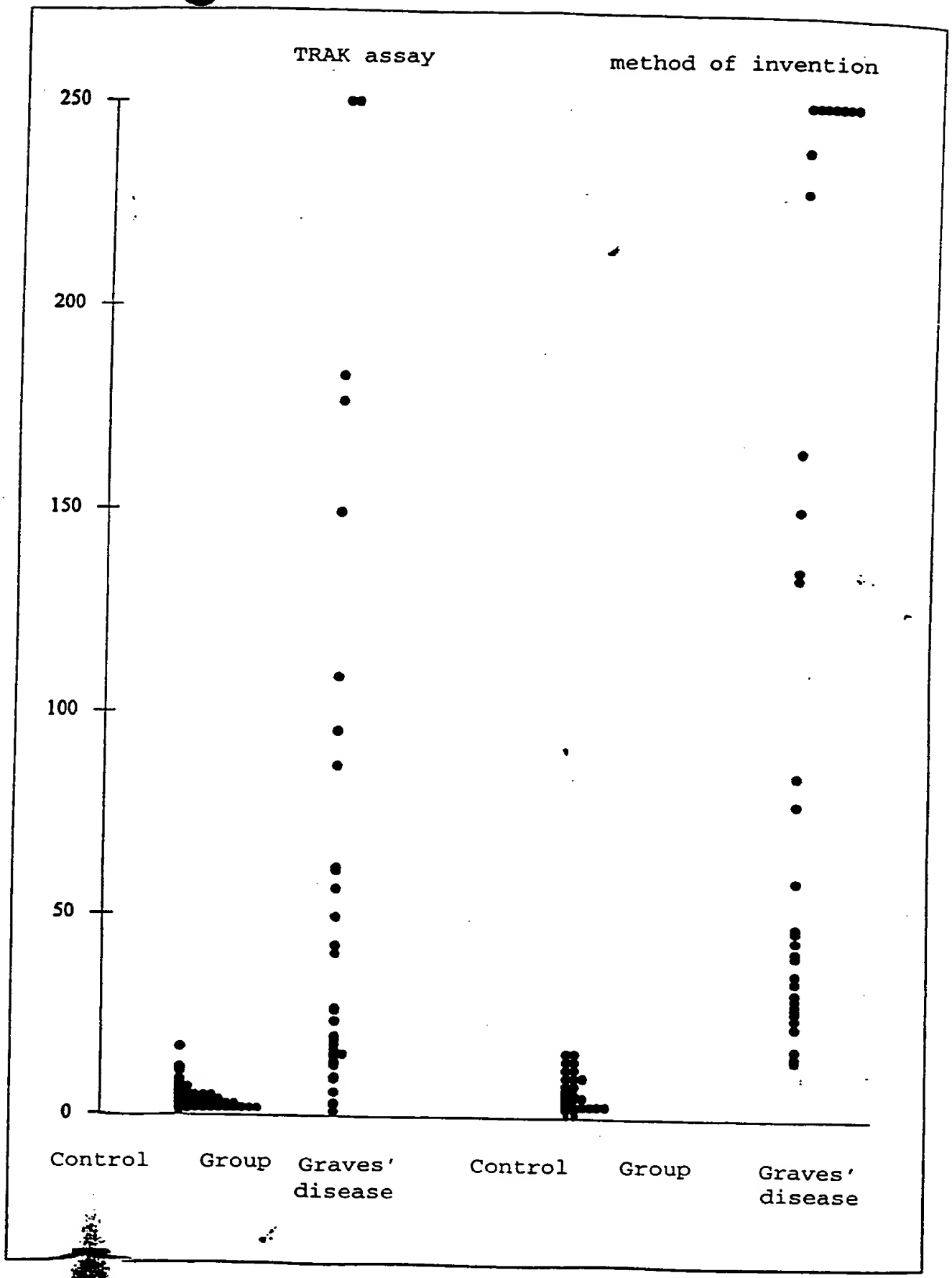
TRAK assay
method of invention
250
200
150
100
50
0
Control
Group
Graves' disease
Control
Group
Graves' disease

Fig.